AD_____

Award Number: DAMD17-01-1-0570

TITLE: Control of Metastasis-Associated Gene Expression by Cell-Surface Beta-1, 6 Branched Oligosaccharide Expression

PRINCIPAL INVESTIGATOR: William G. Chaney, Ph.D.

CONTRACTING ORGANIZATION: University of Nebraska Medical Center
Omaha, Nebraska 68198-6810

REPORT DATE: June 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Final (1 Jun 01 -31 May 02)

4. TITLE AND SUBTITLE
Control of Metastasis-Associated Gene Expression by Cell-Surface Beta-1, 6 Branched Oligosaccharide Expression

5. FUNDING NUMBERS
DAMD17-01-1-0570

6. AUTHOR(S):
William G. Chaney, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Nebraska Medical Center
Omaha, Nebraska 68198-6810

E-Mail: spadmin@unmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Metaststic breast cancer cells express cell-surface Beta-1,6 branched oligosaccharide structures, which are reduced/absent in normal breast tissue. The biosynthesis of these structures is initiated by the Golgi-localized glycosyltransferase N-Acetylglucosaminyl-trasnferase V (GlcNAc-T V). The altered expression of GlcNAc-T V in animal mammary cancer models also influences metastasis. Furthermore, altered transcription of metastasis-associated genes has also been observed in cells with a reduced expression of cell-surface Beta-1,6 branched oligosaccharide expression.

The aim of this project is to characterize the altered expression of mRNA in mouse and human mammary cancer-derived cell lines as a function of altered Beta-1,6 branched oligosaccharide expression. This will be accomplished by using DNA Microarray technology to assess the mRNA levels in cell lines transfected with GlcNAc-T V expression vectors. After identification of mRNA molecules that are altered by changes (elevation or reduction) in cell-surface Beta-1,6 branched oligosaccharide expression, the kinetics of induction will be characterized using cell lines with GlcNAc-T V under the control of an inducible promoter.

14. SUBJECT TERMS
oligosaccharides, glycosyltransferases, DNA microarrays, gene expression, metastasis

15. NUMBER OF PAGES
10

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Elevated levels of Beta-1,6 branched oligosaccharides has been associated with the development of metastatic lesions, both in human breast cancer and in murine mammary cancer models of this disease. The levels of Beta-1,6 branched oligosaccharides are controlled by the glycosyltransferase N-Acetylglucosaminyl-transferase V (GlcNAc-T V). Previous reports have demonstrated that changes in cell-surface Beta-1,6 branched oligosaccharide levels are accompanied by changes in metastasis-associated genes at the transcriptional level. The purpose of this research is to determine the genes that are altered in their expression at the mRNA level by changing the cell-surface expression of Beta-1,6 branched oligosaccharides. This will be accomplished by using DNA Microarray technology to scan thousands of genes for altered expression using mouse mammary and human breast cancer cell lines that have been transfected with an expression vector for GlcNAc-T V.

This laboratory has created cell lines with altered expression of cell-surface Beta-1,6 branched oligosaccharides(1-3). Two approaches have been taken to obtain these modifications of cell-surface Beta-1,6 branched oligosaccharide expression. To reduce the biosynthesis of these determinants, cells were selected for survival in cytotoxic concentrations of the plant lectin, L-PHA, which binds to the Beta-1,6 branched oligosaccharide structures(1,2). These cell lines show a reduced level of metastasis when examined in an experimental metastasis assay by determining the levels of lung tumor formation following injection into the tail veins of mice (Table 1).

Table 1. Effect of Reducing Cell-Surface Beta-1,6 Branched Oligosaccharides on Metastasis

Cell Line	Lung Tumors
S2(parent)	85.6 ± 15.5
S2-R1	9.3 ± 3.5
S2-R2	31.8 ± 7.1
S2-R8	15.6 ± 9.6
168.1(parent)	11.3 ± 3
L1.11	3.0 ± 1
L1.12	1.0 ± 0
L1.14	2.0 ± 0

- Cells(10^5) in 0.1 ml PBS were injected into the tail vein of mice and the lung tumors measured after three weeks. Data taken from ref. 1 and 2.

To elevate the levels of these molecules, cells were transfected with an expression vector containing the cDNA for GlcNAc-T V(3). The resulting expression of this glycoslytransferase resulted in an elevation in Beta-1,6 branched oligosacchride expression. These cells had an increased expression of cell-surface Beta-1,6 branched oligosaccharide expression and a subsequent elevation in lung tumor formation when injected into the tail vein of mice in the experimental metastasis assay. These cell lines provide the tools for the evaluation of the effects of altering the expression of Beta-1,6 branched oligosaccharides on glycoproteins at the cell surface on gene expression at the mRNA level.

Table 2. Effect of Elevating Cell-Surface Beta-1,6 Branched Oligosaccharide Expression on Tumor Cell Metastasis.

Cell Line	Lung Tumors
168.1-C1	1 + 1
168.1-TV.24	53 + 31
66.1-C2	32 + 5
66.1-TV18	119 + 19
410.4-C6	3 + 1.5
410.4-TV.84	92 + 11

Cells (2×10^4) in 0.1 ml PBS were injected into the tail vein of mice. Cells designated with a "C" were transfected with the control empty vector, whereas those designated "TV" with the GlcNAc-T V expression vector causing overexpressed GlcNAc-T V. aand an elevation of cell-surface Beta-1,6 branched oligosaccharide expression. Lung tumors were measured three weeks later. Data taken from ref. 3.

Body

The observations of Dennis and coworkers that lowered cell-surface Beta-1,6 branched oligosaccharide expression was associated with alterations in the transcription of metastasis-associated genes in a manner consistent with the reduced metastatic properties of the cells(4) led to the present hypothesis by this laboratory that a novel mechanism exists in cells by which the alterations in Beta-1,6 branched oligosaccharide expression could result in the alteration of the transcription of metastasis-associated genes.

In these experiments total RNA has been purified from cells with elevated levels of GlcNAc-T V. Two types of cells have been used. The first is the murine mammary cancer cell lines described in Table 2 that have been transfected with either a control vector or an expression vector containing GlcNAc-T V under the control of the CMV promotor. These cell lines have been characterized and found to have a stable expression of elevated levels of cell-surface Beta-1,6 branched oligosaccharides. The second cell line is the human kidney 293 cell line that has the expression of GlcNAc-T V under the control of the ecdysone-inducible promotor. Treatment of these cells with Ponasteone A, an ecdysone analogue, results in an elevation in cell-surface Beta-1,6 branched oligosaccharide expression.

Cell Growth. Cells were grown in RPMI 1640 media containing 10% fetal bovine serum. Cells were harvested when they were 60-80 percent confluent. Cells with the inducible GlcNAC-T V vector (22.03 cell line) were treated with 1.0 M Ponasterone A for 40 hr. prior to RNA preparation.

RNA Purification. RNA was purified by the guanidine-thiocyanate-phenol method using Trizol reagent(Invitrogen corp.). Following this treatment, the RNA was separated from residual genomic RNA. In all samples the A260/A280 ration was between 1.0 and 2.0, indicating a lack of contamination with protein. Electrophoretic analysis on 1% agarose gels in Tris-Acetate-EDTA buffer was performed on all samples to characterize the purity of the RNA preparations with respect to contamination with genomic DNA, seen as a high molecular weight band near the top of the gel and degraded RNA, seen as a band near the dye front. All samples used for subsequent analysis were free of genomic DNA and low molecular weight RNA species.

DNA Microarray Analysis. Purified RNA samples were taken to the UNMC DNA Microarray Core Facility. Paired samples purified from experimental and control cell lines on the same days and at similar confluence were chemically labelled with Cy3 or Cy5 fluorescent dyes and used to probe DNA microarray plates prepared in the facility. A panel of 2300 cDNA species synthesized in the facility from cDNA clones was used in these experiments. Experiments utilizing mouse cells used a mouse cDNA containing chip and those using the human DNA used a human cDNA chip. Ratios of hybridization of experimental and control labeled RNA's was computed from the chip image using GenePixPro software. Data from spots on the array giving unreliable results as indicted by the program were eliminated from the analysis.

The murine cell line 410.4-C6 and its GlcNAc-T V transfected partner, 410.4-TV.84 were tested. The identity of clones that were positive when these cells were tested on two different days from two different mRNA preparations isolated on different days. Table 3 shows the identity of genes that were reduced by greater than two-fold in the GlcNAc-T V transfected cells.

Table 3. Genes Inhibited by Elevated Beta-1,6 Branched Oligosaccharide Expression

Gene Name	Accession Number
	AA407367
Mus musculus ubiquitin specific protease	AA407699
	AA407948
Mus musculus caspase 8 associated protei	AU017543
Mus musculus caspase 8 associated protei	AU022192
L.japonicus gene encoding RING finger pr	AU022729
R.norvegicus fos-related antigen DNA, ex	AU042525
Mus musculus caspase 12 (Casp12), mRNA	AW552373
Mus musculus BCL2/adenovirus E1B 19 kDa-	AW553554
Homo sapiens eukaryotic translation init	AW554208
Mus musculus complement component 1, q	BG062931
	BG063041
Mus musculus adrenomedullin (Adm), mRNA	BG063462

RNA from cells bearing transfected GlcNAc-T V was compared by microarray analysis with that of control transfected cells. Genes inhibited two-fold or greater in two independent experiments are shown.

Genes elevated two-fold or greater in the 410.4-TV.84 cells were also identified. Those genes elevated in the two separate experiments are listed in Table 4.

Table 4. Genes Induced by Elevated Beta-1,6 Branched Oligosaccharide Expression

Gene Name	Accession Number
Mus musculus cyclin D1 (Ccnd1), mRNA	AU015041
M.musculus p53 gene, bending region	AU019816
Mus musculus transforming growth factor,	AU022195
igfbp-4=insulin-like growth factor-binding	AU041376
Mouse kidney ornithine decarboxylase mRNA	AW537017
	BG063482

RNA from cells bearing transfected GlcNAc-T V was compared by microarray analysis with that of control transfected cells. Genes showing a two-fold or greater increase in expression in two independent experiments are shown.

The second cell line to be analyzed for the effects of elevated Beta-1,6 branched oligosaccharide expression is the 22.03 cell line. It has been created in this laboratory by transfecting Ecr-293 cells (the human kidney cell line transfected with the ecdysone receptor required for activation by ecdysone, or an analogue) the GlcNAc-T V cDNA placed in the pIND expression vector. This cell line was found to have a two-fold elevation of its beta-1,6 branched oligosaccharide expression when treated with an ecdysone analog inducer, Ponasterone A.

The 22.03 cell line was split into two flasks and treated for two days with either 1.0 mM Ponasterone A or the carrier alone. Cell density at the end of the experiment was 80% for both cell preparations. Following RNA isolation and Microarray analysis was performed as described above, but using a human cDNA gene chip containing 2300 genes. Table 5 lists those genes that were reduced as a result of the induction of GlcNAc-T V synthesis. Table 6 lists those that were elevated with GlcNAc-T V induction.

Table 5. Genes Reduced in 22.03 Cells after Induction of GlcNAc-T V.

hexabrachion (tenascin C, cytotactin)
putative tumor suppressor
DKFZP564I122 protein
thyroxin-binding globulin
interleukin 1 receptor antagonist
heme oxygenase (decycling) 1
CD48 antigen (B-cell membrane protein)
structure specific recognition protein 1
melanoma antigen, family A, 10
glucose regulated protein, 58kD
zinc finger protein 238
integrin, alpha L (antigen CD11A (p180),
neuronal pentraxin I
LIM domain kinase 1
thrombomodulin
protein tyrosine phosphatase, receptor t
putative transmembrane protein
interleukin 2 receptor, gamma (severe co
apolipoprotein C-III
coatomer protein complex, subunit alpha
tumor necrosis factor receptor superfami
protease, metallo, 1, 33kD
dedicator of cyto-kinesis 2
myeloid/lymphoid or mixed-lineage leukem
upstream binding transcription factor, R
nephronophthisis 1 (juvenile)
tumor necrosis factor receptor superfami
tumor necrosis factor, alpha-induced pro
Human clone 23627 mRNA, complete cds

Table 6. Genes Reduced in 22.03 Cells after Induction of GlcNAc-T V.

amyloid beta (A4) precursor protein-bind
B-cell CLL/lymphoma 6 (zinc finger prote
CD36 antigen (collagen type I receptor,
CD3D antigen, delta polypeptide (TiT3 co
CD79B antigen (immunoglobulin-associated
Cerebellin 1 precursor
Chitinase 1 (chitotriosidase)
Collagen, type VI, alpha 3
ESTs, Weakly similar to PUTATIVE RHO/RAC
ESTs, Weakly similar to similar to ribos
GDNF family receptor alpha 2
H. Sapiens mRNA for cytokeratin 20
Homo sapiens transcriptional enhancer fa
Human clone 23909 mRNA, partial cds
Interferon-induced protein 41, 30kD
Interleukin 1 receptor-like 1
Interleukin 7 receptor
Plasminogen
Pleckstrin homology, Sec7 and coiled/coi
Polycystic kidney disease 2 (autosomal d
Sarcolipin
Sialyltransferase 8 (alpha-N-acetylneura
Tissue factor pathway inhibitor 2
Transferrin
Transketolase (Wernicke-Korsakoff syndro
Troponin I, skeletal, fast
Ubiquitin specific protease 11
Uteroglobin
Vascular cell adhesion molecule 1
Zinc finger protein 151 (pHZ-67)
Zinc finger protein 35 (clone HF.10)

RNA from 22.03 cells treated for 48 hr with Ponasterone A was analyzed for elevation of gene expression in comparison with uninduced cells.

Key Research Accomplishments.

Murine genes whose synthesis is reproducibly inhibited by elevated Beta-1,6 branched oligosaccharide expression have been identified.

Murine Genes whose synthesis is reproducibly induced by elevated Beta-1,6 branched oligosaccharide expression have been identified.

Human genes whose synthesis is inhibited by elevated Beta-1,6 branched oligosaccharide expression have been identified.

Human genes whose synthesis is induced by elevated Beta-1,6 branched oligosaccharide expression have been identified.

Reportable outcomes.

None yet, although this work will lead to publications and future grant applications.

Conclusions.

At the outset of this concept award the hypothesis to be tested was that altering cell-surface Beta-1,6 branched oligosaccharide expression could have an effect on gene expression at the mRNA level. How changing the carbohydrate modification at the cell surface, especially the branching of the Asparagine-linked oligosacchrides at the cell surface could affect gene expression at the transcriptional level is not know. But, the data gathered by this project confirms that cell lines that have had an elevation in GlcNAc-T V, causing an elevation of Beta-1,6 branched oligosaccharide expression, show changes in the RNA levels of a number of genes. Using a murine mammary cancer cell model, both elevation and reduction in gene expression is observed. Furthermore, changes in gene expression were found in human cells as a function of GlcNAc-T V levels. Human breast(and colon, esophogael, and pancreatic) cancers have elevated Beta-1,6 branched oligosaccharide levels in tumors found *in vivo*. Increased expression of Beta-1,6 branched oligosaccharides correlates with tumor progression. But the functional role that Beta-1,6 branched oligsaccharide expression might play in tumor development is unclear. These results demonstrate that one way these structures might contribute to the development of malignant metastatic tumors is to alter gene expression in cells. The mechanism by which this could occur is unknown, and completely novel. As well as determining important factors in breast cancer development, this work will lead to the study of presently unknown pathways for the regulation of gene transcription.

References.

1. Lu, Y., J. Pelling, and W. Chaney(1994). Tumor cell surface Beta-1,6 Branched Oligosaccharides and Lung Metastasis. Clin. Exp. Metastasis, 12:47-54.
2. Lu, Y.(1992) Ph.D. Thesis, University of Nebraska Medical Center.
3. Seberger, P, and W. Chaney(1999). Control of Metastasis by Asn-linked Beta-1,6 Branched Oligosaccharides. Glycobiology, 9:235-241.
4. Korczak, B., and J. Dennis (1993). Inhibition of N-linked Oligosaccharide Processing in Tumor Cells is Associated with Enhanced Tissue Inhibitor of Metaloproteinases (TIMP) Gene Expression. Int. J. Cancer. 53:634-639.

Personnel

Jennifer Green